Exercise intensity modulates the appearance of circulating microvesicles with pro-angiogenic potential upon endothelial cells

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Abstract

The effect of endurance exercise on circulating microvesicle dynamics and their impact upon surrounding endothelial cells is unclear. Here we tested the hypothesis that exercise intensity modulates the time-course of platelet (PMV) and endothelial-derived (EMV) microvesicle appearance in the circulation through haemodynamic and biochemical-related mechanisms, and that microvesicles formed during exercise would stimulate endothelial angiogenesis *in vitro*. Nine healthy young men had venous blood samples taken prior, during and throughout the recovery period after 1 h of moderate (46 ± 2% $\dot{V}O_2$max) or heavy (67 ± 2% $\dot{V}O_2$max) intensity semi-recumbent cycling and a time matched resting control trial. *In vitro* experiments were performed by incubating endothelial cells with rest and exercise-derived microvesicles to examine their effects on cell angiogenic capacities. PMVs ($CD41^+$) increased from baseline only during heavy exercise (from 21 ± 1 x 10^3 to 55 ± 8 x 10^3 and 48 ± 6 x 10^3 PMV / µl at 30 and 60 min, respectively; $P < 0.05$), returning to baseline early in post-exercise recovery ($P > 0.05$), whereas EMVs ($CD62E^+$) were unchanged ($P > 0.05$). PMVs were related to brachial artery shear rate ($r^2 = 0.43$) and plasma noradrenaline concentrations ($r^2 = 0.21$) during exercise ($P < 0.05$). Exercise-derived microvesicles enhanced endothelial proliferation, migration and tubule formation compared to rest microvesicles ($P < 0.05$). These results demonstrate substantial increases in circulating PMVs during heavy exercise and that exercise-derived microvesicles stimulate human endothelial cells by enhancing angiogenesis and proliferation. This involvement of microvesicles may be considered a novel mechanism through which exercise mediates vascular healing and adaptation.

Keywords: platelet microvesicles; microparticles; shear stress; human umbilical vein endothelial cell; angiogenesis

News and Noteworthy

Increases in intravascular [PMV] occur during exercise but this depends on exercise intensity, and correlates with elevations in vascular shear stress and plasma [noradrenaline]. Circulating microvesicles isolated from exercising humans display pro-angiogenic potential upon cultured endothelial cells. Thus, it is possible that microvesicles are involved in vascular responses to exercise.

List of abbreviations

CON, rest control trial; EMV, endothelial-derived microvesicle; eNOS, endothelial nitric oxide synthase; exMVs, circulating exercise microvesicles; HI, heavy intensity trial; HUVECs, human umbilical vein endothelial cells; MI, moderate intensity trial; MTT, thiazol blue tetrazolium bromide; PMV, platelet-derived microvesicle; PPP, platelet poor plasma; rMVs, circulating rest microvesicles; $\dot{Q}$, cardiac output; SR, shear rate; SV, stroke volume; $\dot{V}CO_2$, carbon dioxide output; $\dot{V}O_2$, oxygen uptake; $\dot{V}O_2$max, maximal oxygen uptake; VT1, first ventilatory threshold.

Introduction

Intraluminal shear stress is a key modulator of endothelial nitric oxide synthase (eNOS) activity and expression in arteries (45, 61), and has been identified as a major stimulus leading to the exercise-induced improvement in vascular function (4, 13, 52, 53), but endothelial adaptations also take place at sites not directly exposed to increased shear forces and acute changes in circulating factors are believed to be involved in such adaptations (43). However, the mechanisms by which exercise bring about endothelial adaptations not directly dependent on shear stress are not fully understood and may relate to a variety of still unidentified factors.

Cell-derived microvesicles have been identified as potential biomarkers linked to obesity (19), dyslipidaemia (1), and coronary artery disease (3, 25). In addition, increased concentrations of several microvesicle varieties are found in the blood post exercise (11, 31, 34, 48, 49). As small membrane-derived vesicles (diameter < 1 μm), their phenotype depends on their cell of origin and stimulus of formation (29, 47). Platelet-derived microvesicles (PMVs) are the most abundant circulating microvesicle population (24, 48, 49), and *in vitro* stimulation of platelets with thrombin (7), noradrenaline (54), IL-6 (42), and shear stress (39, 44) induce shedding of PMVs, whereas high shear stress downregulates the shedding of endothelial microvesicles (EMVs) from cultured endothelial cells (56).

Plasma PMV appearance occurs during recovery from maximal (11) and submaximal (34, 36, 48, 49) exercise protocols, whereas a less pronounced increase or completely absent alteration in plasma EMV (31, 34, 48, 49), erythrocyte (11) and monocyte microvesicles (11, 48, 49) has been observed. The dynamics of PMV during exercise, however, is unknown and the impact of exercise intensity on circulating microvesicle appearance has not been explored. Several intravascular factors known to stimulate *in vitro* PMV and EMV production (29, 39, 44, 54) are modulated by exercise intensity. Because vascular shear stress is expected to increase with workrate, and elevated shear forces are strong agonists stimulating *ex vivo* microvesicle blebbing from platelets, we anticipated that PMVs would increase during exercise according to exercise intensity, whereas EMV release would be restricted by elevated shear stress (56).

Beyond being simple inert plasma membrane fragments, microvesicles are now recognised as bioactive blood constituents capable of interacting with diverse cell populations, including the vascular endothelium (47). Certain EMVs isolated from cell culture exhibit paracrine effects; stimulating inflammation, increasing superoxide anion production while decreasing nitric oxide bioavailability in recipient endothelial cells (8, 14). Depressed endothelial function has also been

reported in arteries exposed to circulating microvesicles from cardiac patients and pre-eclamptic women, but not from apparently healthy donors (6, 55). In contrast, PMVs may protect endothelial cells from apoptosis (30) and stimulate angiogenesis *in vitro* (7, 30) and *in vivo* (7). The physiological relevance of microvesicles produced with exercise upon the vascular endothelium, however, has not been explored but based on previous research we postulated that microvesicles formed during exercise bouts could be involved in vascular adaptations, leading to a proliferative and angiogenic endothelial phenotype.

Therefore, we aimed (1) to characterize the impact of modulating exercise intensity on the time-course of PMV and EMV concentrations during exercise and recovery; (2) to examine putative relationships between shear stress and microvesicle formation during exercise *in vivo*; (3) and to investigate the potential impact of circulating microvesicles produced during exercise on angiogenesis, proliferation, migration, of human endothelial cells. We hypothesised that PMVs, but not EMVs, would increase during exercise with intensity, and remain elevated up to 1 h post-exercise; that the increase in PMVs would be associated with shear stress during exercise; and that exercise-derived circulating microvesicles would stimulate endothelial cell proliferation, migration and angiogenesis *in vitro* to a greater extent than those isolated at rest.

Methods

Ethical approval

Written informed consent was obtained from all participants prior taking part in the experiments. Human and cell culture experimental procedures were approved by Brunel University London Research Ethics Committee (RE55-12 and RE39-13), and experiments were performed in accordance with the Declaration of Helsinki.

Participants

Nine healthy young participants (25 ± 1 yr, 1.79 ± 0.03 m, 80.5 ± 4 kg, $\dot{V}O_2max$ 3.3 ± 0.2 l / min^{1}) completed all five visits of this study. Volunteers were young lean male non-smokers, who were free from cardiorespiratory and metabolic diseases, and mostly involved in recreational physical activities (self-reported). Microvesicles used in the cell culture experiments were obtained from samples collected from a subset of this group (n = 6; 24 ± 1 years, 1.79 ± 0.04 m, 82.5 ± 5.9 kg, $\dot{V}O_2max$ 3.3 ± 0.3 l / min).

Experimental design

120 Two experiments were performed to (1) elucidate the time-course of microvesicle appearance in the
121 circulation in response two intensities of dynamic exercise, while gaining insights about the potential
122 mechanisms involved with this response *in vivo*; and to (2) investigate the impact of circulating
123 microvesicles produced during exercise upon endothelial proliferation, chemotaxis, and
124 angiogenesis *in vitro*.

125 All participants attended the laboratory for 2 preliminary visits, followed by 3 experimental trials
126 during which exercise or resting control experiments were performed in a thermoneutral
127 environment. Participants were familiarised with the experimental procedures, the semi-recumbent
128 cycle ergometer (Angio, Lode, Netherlands) and the maximal incremental exercise test at the first
129 visit to minimise possible learning effects. After at least 48 h of recovery, participants had their
130 oxygen uptake ($\dot{V}O_2$), CO_2 output ($\dot{V}CO_2$) and derived variables determined using a ramp incremental
131 test with continuous collection of expired gases using open-circuit spirometry (Quark B^2, Cosmed,
132 Italy). Briefly, the test started with participants cycling at 80 rpm at a power output of 25 W with a
133 ramp slope of 25 W / min. They cycled continuously until the limit of exercise tolerance, and a
134 confirmatory step test to exhaustion was performed after 5 min of recovery at a relative intensity 5%
135 above their ramp peak power output (46). Data were analysed using 10 s moving average windows
136 and the maximal oxygen uptake ($\dot{V}O_2$max) was determined as the highest $\dot{V}O_2$ obtained during the
137 incremental test. The first ventilatory threshold (VT1) was determined using a two criteria
138 confirmation method, comprising the first deflection point in the $\dot{V}O_2$–$\dot{V}CO_2$ curve, and the point
139 when the ventilatory equivalent of O_2 increased without a concomitant rise in the respective CO_2
140 equivalent (9).

141 The last three visits were the experimental trials. Participants arrived at the laboratory in the
142 morning, approximately 1.5 h after a light breakfast and rested for approximately 30 min during
143 instrumentation. The first measurement took place approximately 20 min after cannulation. During
144 the resting control trial (CON) participants remained in a semi-recumbent position throughout the
145 protocol (240 min) while blood samples were obtained at baseline, 30, 60, 80, 100, 120, 150, 180
146 and 240 min of the protocol. Haemodynamic measurements were obtained at similar time-points.
147 Exercise trials were performed in random order. For the moderate intensity exercise trial (MI), 60
148 min of cycling at 80% of the workrate relative to VT1 (*i.e.* 46 ± 2% of $\dot{V}O_2$max) was performed
149 followed by 180 min of rest (recovery period), whereas during the heavy exercise intensity trial (HI)
150 participants cycled at 30% of the difference between the workrate relative to their individual VT1
151 and $\dot{V}O_2$max (*i.e.* 67 ± 2% of their $\dot{V}O_2$max). The delta method was selected for the HI protocol
152 because it reduces the variability of inter individual physiological responses (35), and the

classification of exercise domains as moderate and heavy intensity was based on previous work (60). The three experimental trials were separated by at least one week to reduce potential cumulative effects of blood sampling.

The second set of experiments investigated the impact of exercise-derived microvesicles on endothelial cells. Culture studies were performed by incubating human umbilical vein endothelial cells (HUVECs) with circulating microvesicles obtained from a subset of the previous experimental group at rest (rMVs) or during heavy exercise (exMVs). Endothelial cell capacity to migrate, proliferate, repair a disturbed monolayer, and form tubule-like structures *in vitro* was assessed. Experiments were performed by supplementing the experimental media with microvesicles. FBS or VEGF was used as positive control, and the supernatant of microvesicle pellets (microvesicle free plasma) was used as an internal control.

Human experiments

Catheter placement and blood sample storage

Venous blood samples were taken during the CON, MI and HI trials. Upon arrival, participants rested in the supine position in a quiet room and an 18 gauge cannula (BD Venflon, Becton, Dickson and Company, USA) was inserted into a superficial antecubital vein of the arm. A 0.9% NaCl solution (BD PosiFlush, Becton, Dickson and Company, USA) was flushed through the cannula to maintain patency following each blood draw. Samples were obtained without venous stasis and the first 3 ml of blood were discarded. Collected blood was immediately mixed in tubes containing 0.129 mol / l sodium citrate (Sigma-Aldrich, USA) or 8 mg of EDTA (Sarstedt, Germany). Hct, Hb, and lactate were determined in whole blood samples. Platelet rich plasma was obtained by centrifugation at 300 x g at 4°C for 10 min. EDTA samples were aliquoted in tubes and stored at -80 °C. Sodium citrate plasma underwent a second centrifugation step at 15,000 x g at 4°C for 10 min to obtain platelet poor plasma (PPP). Aliquots were stored at -80 °C for microvesicle quantification or further cell culture experiments.

Systemic and Limb Haemodynamics

Arterial blood pressure and heart rate (HR) were recorded continuously using a Finometer® Pro device (Finapress Medical Systems, Netherlands) throughout the 3 experimental trials. Participant's stroke volume (SV) was determined from apical four chamber echocardiography (phased array probe, Vivid 7, GE, UK). Measurements were obtained with participants in the semidecubitus position on their left side, which was accomplished by tilting the recumbent cycle ergometer. End diastolic and end systolic left ventricular areas were determined using EchoPac software (version

112, GE, UK) and volumes calculated using the single plane Simpson's method (33). At least three heart cycles were selected for analysis. Cardiac output ($\dot{Q}$) was calculated as SV multiplied by the corresponding HR. $\dot{Q}$ as well as SV were estimated from the Finometer® Pro in situations where the echocardiographic recordings could not be obtained or were of low quality. $\dot{Q}$ of 5 participants obtained during diverse resting and exercise conditions displayed a moderate agreement between echocardiographic and Modelflow methods, with the mean difference of -0.5 l / min not being different from 0 (single sample T test $P > 0.05$; 95% confidence intervals: 4.1 to -5.2), and no proportional bias ($P > 0.05$).

Upper and lower limb haemodynamics were assessed by vascular ultrasonography with a multi-frequency linear array transducer (Vivid 7 ultrasound, GE, UK) to examine the relationship between haemodynamic changes and circulating microvesicle concentrations. The left brachial artery was assessed at rest and during leg exercise. Blood flow was calculated as the product of Vmean x π x (D^2 / 4) x 60; and vascular shear rate (SR) was calculated as 4 x Vmean / D, where Vmean refers to weighted time averaged mean Doppler velocity, and D refers to vessel diameter (4, 53). The common femoral artery was assessed at baseline and throughout recovery, but it could not be assessed during exercise due to the dynamic nature of cycling. Hence, leg blood flow during exercise was estimated assuming two leg blood flow = exercise $\dot{Q}$ - (resting $\dot{Q}$ + exercise two arm blood flow).

Microvesicle quantification

Platelet poor plasma samples were thawed at room temperature and Fc receptors were blocked with Human TruStain FcX™ (BioLegend, USA) for 10 min. Samples were incubated in the dark for 25 min with PE conjugated anti-human CD62E (E-selectin; BioLegend, USA; at 18 µg / ml), and PE/Cy5 anti-human CD41 (integrin α2b; BioLegend, USA; at 2.04 µg / ml) fluorescent antibodies. Samples were diluted 1:40 in 0.2 µm filtered PBS and centrifuged for 15 min at 17,960 x g and 4°C. The microvesicle pellet was resuspended in PBS. Microvesicle concentration was quantified using an ImageStream^{X}® Mark II imaging flow cytometer (Amnis Corporation, USA) using INSPIRE software (Amnis Corporation, USA), with 60x magnification. All events were collected and sample acquisition was limited to no less than 1,000 events in the expected PMV gate. The *a priori* choice for PMV gate relied on previous evidence indicating that PMVs are more responsive to an exercise stimulus than EMVs. Single-stained samples were used to create a multi-staining compensation matrix.

Data were analysed off-line with IDEAS software (version 6.1, Amnis Corporation, USA) using an adapted microvesicle gating strategy described by others (24). Briefly, after compensation, microvesicles were determined as events below 1 µm diameter using calibration beads (Fluoresbrite,

Polysciences, USA), and displayed very low side scatter and moderate mean fluorescence intensity for PE/Cy5 (PMVs), or PE (EMVs) (Figure 1) (24). A threshold for positive events was set as adapted from the fluorescence minus one procedure.

Plasma noradrenaline and interleukin-6 quantification

Plasma noradrenaline and IL-6 concentration were measured in EDTA samples by commercial ELISA kits (Noradrenalin ELISA, IBL International, Germany; Human IL-6 Quantikine, R&D Systems, USA). Samples were analysed in duplicate according to the manufacturer's instructions. The mean coefficient of variation between duplicates was 9.7% for noradrenaline and 6% for IL-6.

Whole blood haematocrit, haemoglobin, lactate, and plasma volume corrections

One ml of blood was immediately separated to determine Hct, Hb, and whole blood lactate concentrations. Hct was determined by packed cell volume method after standard centrifugation of sodium-heparinized capillary tubes (micro-haematocrit tubes, HaematoSpin 1400 centrifuge, Hawksley, UK). Blood Hb concentration was obtained by photometric analysis (HemoCue® Hb 201+ System, HemoCue AB, Sweden), and lactate concentration was determined using a Biosen C-line analyser (EKF Diagnostics, UK) after daily calibration. Plasma volume shifts were calculated as described previously (17).

Cell culture experiments

Culture of human umbilical vein endothelial cells

HUVECs were cultivated in culture flasks (T75, Sarstedt, Germany) in growth medium (medium 199 supplemented with 20% FBS, 15 µg / ml endothelial cell growth supplement, 5 U / ml of heparin, 100 U / ml - 100 µg / ml of penicillin-streptomycin, 0.6 mg / ml pyruvate, and 20 mM HEPES), at 37°C in 5% CO_2, with half medium changes every 48 – 72 h. Cells were removed from wells with trypsin when monolayers became 60 – 80% confluent for passaging or experiments. Experiments were performed with cells between passage 3 – 6.

Microvesicle experimental medium preparation

On the day of the experiments, PPP vials were thawed at room temperature, diluted 1:1 in PBS, and isolated by centrifugation for 1 h at 17,500 g at 4 °C as adapted from others (6, 55). Experimental media were prepared by suspending the microvesicle pellet to its original concentration in FBS free growth medium for migration and wound-healing assays, or to double the original microvesicle concentration in medium 199 supplemented with penicillin-streptomycin and heparin for

proliferation or tubule formation assays (diluted to the original concentration in subsequent steps of those corresponding assays). Thus, microvesicles were administered at their physiological concentrations determined in plasma samples at baseline (*e.g.* 24.1 x 10^3 PMV / µl (25.9, 20.0), and 12.4 x 10^3 EMV / µl (14.3, 10.6); median (96% confidence intervals), n = 5 per assay) or during exercise (*e.g.* 49.9 x 10^3 PMV / µl (63.7, 41.8), and 12.7 x 10^3 EMV / µl (14.3, 10.5); median (96% confidence intervals), n = 5 per assay). The plasma supernatant obtained after centrifugation was used to make separate aliquots of microvesicle free experimental media. Results from a pilot study confirmed very low concentrations of microvesicles in these plasma supernatant samples (*i.e.* 9 ± 2% positive microvesicle events in comparison to the microvesicle pellet suspension; n = 4). Hence, microvesicle free plasma experimental medium was used as an internal control in the current experiments.

Proliferation assay

Proliferation of HUVECs was quantified using the thiazol blue tetrazolium bromide (MTT) assay in 0.5% gelatin pre-coated 96 well plates. A total of 50 µl of the cell suspension (2,000 cells) was seeded in each well followed by the addition of 50 µl of experimental medium. Experimental medium with 20% FBS served as positive controls. After 48 h each well was incubated with the MTT solution for a further 4 h. All but 30 µl was removed and the wells were incubated for 10 min in DMSO before absorbance analyses were obtained at 540 nm using a micro plate reader (ELx808, BioTek Instruments, USA). The average of quadruplicates was calculated and expressed as a percentage of the negative control condition.

Migration assay

Migration of HUVECs toward microvesicle containing wells was determined using a modified 48 well Boyden chamber (AP48, Neuro Probe, USA). Experimental medium (rMVs, exMVs, or microvesicle free supernatant) was loaded into lower chamber wells to form a slight meniscus, then a pre-coated gelatin polycarbonate filter was positioned over the lower chamber. Wells loaded with 10% FBS served as positive controls. The upper chamber was assembled and loaded with 50 µl of a suspension of serum starved cells in microvesicle free medium (25,000 cells), and incubated for 4 h. Adherent cells on the underside of the filter were fixed with methanol and stained with Giemsa. Three images of each well acquired at random locations were obtained with an Axioskop 2 microscope (Zeiss, Germany) at 20x objective magnification, and the number of migrated cells were counted with ImageJ software (version 1.48, National Institutes of Health, USA). The average of triplicates was expressed as a percentage of positive controls.

Scratch wound-healing assay

The scratch wound-healing assay was performed to investigate the impact of microvesicles on HUVECs undergoing the repair process. Cells were seeded on gelatin pre-coated 96 well plates and incubated at 37 °C in 5% CO_2 until cells became almost confluent. On the experimental day and after 4 h of serum starvation, a scratch was made by disturbing the cell monolayer with a 200 µl pipette tip. All medium was aspirated and replaced with experimental medium. Medium supplemented with 20% FBS served as positive control. Images were obtained at baseline and after 3.5 h of incubation using an inverted microscope (Axiovert 200M, Zeiss, Germany) at 10x objective magnification. The cell free area was measured using ImageJ software (version 1.48, National Institutes of Health, USA), and the wound closure was calculated by subtracting the cell free area obtained post incubation from the baseline measurement. The average of quadruplicates for each condition was expressed as a percentage of the wound closure observed in the negative control condition.

Tubule formation assay

The angiogenic potential of microvesicles was assessed by a tubule formation assay. Briefly, 96 well plates were coated with basement membrane protein gel (Geltrex®, Thermo Fisher Scientific, UK) on ice at 78 µl per cm^2. The gel was gently spread with combitip inserts, and the plate was incubated at 37 °C to allow the gel to solidify (20). Serum starved cells were suspended in experimental medium 199 (with penicillin-streptomycin and 0.5% FBS), and 50 µl of cell suspension (10,000 cells) were incubated in Geltrex® pre-coated wells with 50 µl of experimental medium. VEGF (50 ng / ml) was used as positive control. Wells were imaged after 24 h of incubation using an AxioVert microscope (Zeiss, Germany) at 5x objective magnification. The number of tubule-like structures, and branching points of 3 fields of view per well were averaged using ImageJ software (version 1.48, National Institutes of Health, USA), and the average of three wells per condition was expressed as a percentage of the results observed in the negative control condition.

List of materials

Medium 199 (Hyclone; CAT# SH30024.01), modified medium 199 (phenol red, l-glutamine, and sodium hydrogen carbonate free; CAT# M3769), MTT (CAT# M5655), VEGF (CAT# RP-75746) were from Fisher Scientific (UK). Geltrex® (CAT# A1413202) and FBS (CAT# 10270-106) were from Life Technologies (UK). Fc receptor blocking solution (Human Trustain FcX; CAT# 422302), PE anti-human CD62E (CAT#322606) and PE/Cy5 anti-human CD41 antibodies (CAT#303708) were from BioLegend (USA); and combitips used for coating plates for angiogenesis assay were from Eppendorf (0.5 ml, CAT# 12674587, Eppendorf, Germany).

311 *Statistical analysis*

312 All data are presented as mean ± SEM. For the first study, dependent variables over time and
313 conditions were compared by two-way repeated measures ANOVA. If a significant F-ratio was
314 observed for time x condition interactions the Dunnett's test for multiple comparisons was
315 performed to compare within-condition results vs baseline values. Between-condition comparisons
316 across time were performed with repeated measure ANOVA with Bonferroni correction. A within
317 subject repeated measures multiple regression was performed to examine the relationship between
318 circulating microvesicles, haemodynamics, and biochemical variables (5). *In vitro* experiments were
319 compared with one-way repeated measures ANOVA, and differences between conditions were
320 identified by the least significant difference when a significant F ratio was observed.

321 Due to technical issues, data of one individual had to be acquired using a different ultrasound
322 system (ProSound SSD5500, Aloka, Japan) during the exercise trials, with no data recorded on the
323 control day. Due to the completely random nature of the missing data, a mean substitution
324 treatment was applied for the missing data (*i.e.* CON trial), instead of the listwise deletion
325 procedure. In addition, Hb results could not be collected in the MI protocol for one participant
326 because of technical limitations, so a similar mean substitution data treatment was used.

327 General statistical analyses were performed using statistical software (SPSS version 20, IBM, USA),
328 with Dunnett's test derived from GraphPad Prism software (version 5.03, GraphPad Software, USA),
329 and the within subject repeated measures multiple regression calculated with SigmaPlot (version 13,
330 Systat Software, UK), always setting the significance level at $\alpha < 0.05$ for all analyses.

331

332 **Results**

333 *Limb and systemic haemodynamics*

334 Estimated leg blood flow increased during cycling as a function of exercise intensity ($P < 0.05$), and
335 measured leg blood flow remained elevated at 5 min of recovery with more pronounced values after
336 HI exercise ($P < 0.05$) (Figure 2C). Accordingly, early recovery femoral artery mean SR was higher
337 than baseline, with greater values after heavy cycling compared to moderate exercise ($P < 0.05$)
338 (Table 1). This blood flow and SR response occurred mostly through a rise in blood velocity to the
339 lower limbs ($P < 0.05$), with arterial vasodilation (increases in conduit vessel diameter) playing a
340 smaller role (Table 1).

During exercise, the brachial artery dilated and mean blood velocity increased ($P < 0.05$) (Table 1), with a resultant rise in arm blood flow during exercise and into early recovery ($P < 0.05$) (Figure 2D). The higher blood flow to upper limbs during heavy compared to moderate exercise was mostly driven by changes in blood velocity, so mean brachial artery SR was augmented during cycling as a function of exercise intensity ($P < 0.05$), and remained elevated at 5 min of heavy exercise recovery ($P < 0.05$), returning to resting values by 20 min.

Cardiac output was elevated throughout cycling in proportion with exercise intensity ($P < 0.05$) (Figure 2A) as a result of increases in HR and SV, with corresponding adjustments in left ventricular end-diastolic and end-systolic volume (Table 2). In the first 5 min of recovery after HI exercise, $\dot{Q}$ remained higher than baseline and CON ($P < 0.05$), whereas it returned to values similar to rest following the MI protocol ($P > 0.05$). Mean arterial pressure increased during exercise reaching higher values during the HI compared to the MI protocol at 40 min ($P < 0.05$) but quickly returned to baseline at the end of exercise. Mean arterial pressure at 5 min of recovery, however, was lower following the HI compared to MI protocol reflecting a tendency for post-exercise hypotension after prolonged heavy exercise (Figure 2B).

Circulating platelet and endothelial-derived microvesicles at rest and during exercise

PMV and EMV concentrations were stable throughout the 4 h of resting control and did not change with moderate exercise ($P > 0.05$). Similarly, EMV concentrations were unaltered by heavy exercise (Figure 3B). Yet, PMV concentrations increased more than 2-fold by 30 and 60 min of heavy exercise ($P < 0.05$, Figure 3A) after which they decreased. However, a second rise above baseline was evident at 60 min of recovery ($P < 0.05$). The PMV area under the time-concentration curve was larger during HI compared to CON and MI trials (154 ± 16 *vs.* 89 ± 6 and 108 ± 9 x 10^3 PMV x h / µl, respectively) ($P < 0.05$). There were no differences in the EMV area under the curve between CON (46 ± 3 x 10^3 EMV x h / µl), MI (46 ± 1 x 10^3 EMV x h / µl) and HI trials (48 ± 3 x 10^3 EMV x h / µl). Changes in plasma volume did not affect the increases in PMV during and after heavy exercise (Supplementary data 1), and thus the aforementioned results represent uncorrected values.

Blood variables

Plasma volume was reduced by between 10-13% during exercise ($P < 0.05$), but returned to resting values early in recovery after moderate exercise ($P > 0.05$) (Table 3), with the change in plasma volume still evident 20 min after heavy exercise ($P < 0.05$). Since changes in plasma volume did not affect the overall microvesicle results (Supplementary data 1), data of the remaining blood-derived variables are also presented without correction. Plasma noradrenaline concentration rose from

baseline levels during heavy cycling ($P < 0.05$), with higher values observed throughout the heavy exercise period compared to resting control, and at 30 min of exercise compared to moderate cycling ($P < 0.05$) (Table 3). Noradrenaline concentration returned to baseline within the first hour of recovery after heavy exercise remaining stable until the end of recovery ($P > 0.05$). No changes from baseline were observed during resting control or moderate exercise ($P > 0.05$). By the end of 1 h of heavy cycling IL-6 concentration increased compared to moderate exercise and resting control ($P < 0.05$) (Table 3) and values remained elevated above baseline throughout recovery. IL-6 concentrations also increased from baseline in the CON and MI trials during the 3rd and 4th h of the protocol ($P < 0.05$), so that at the end of the recovery period no differences were observed between protocols.

Relationship between microvesicles, shear rate, and blood-derived variables in vivo

Modest yet significant correlations were found between PMVs, vascular SR and noradrenaline concentrations when all time-points were investigated ($P < 0.05$, Figure 4A to C). Exploratory analysis revealed that PMV concentrations moderately correlate with BA SR ($R^2 = 0.43$; $P < 0.05$; Figure 4E), estimated femoral artery SR ($R^2 = 0.48$; $P < 0.05$; Figure 4F) and, to a lesser degree, noradrenaline levels ($R^2 = 0.21$; $P < 0.05$; Figure 4G) prior and during exercise, but not during baseline and recovery time-points only ($P > 0.05$). PMVs displayed no significant correlation to IL-6 within these conditions ($P > 0.05$).

Circulating microvesicles increase endothelial cell proliferation and migration

HUVECs proliferated almost 50% quicker than the negative control condition when treated with rMVs, and proliferation almost doubled from control with exMVs (Figure 5A), with smaller effects observed with microvesicle free supernatant conditions. The exMV treatment increased HUVEC proliferation in comparison to the rMV and both the resting and exercise supernatants ($P < 0.05$). rMVs also increased HUVEC proliferation in comparison to resting supernatant, but no more than the exercise supernatant condition ($P > 0.05$). No differences in cell proliferation were observed between resting and exercise supernatants ($P > 0.05$). Both microvesicle and supernatant conditions stimulated HUVEC migration as depicted in Figure 5B. The microvesicle treatments, however, induced substantially more endothelial migration when compared with their respective supernatants ($P < 0.05$). A greater number of cells migrated towards wells loaded with exMVs in comparison to rMVs ($P < 0.05$), but no difference was observed between the two microvesicle free supernatant conditions.

Microvesicles improve endothelial scratch wound-healing

HUVECs treated with supernatants displayed an almost 2-fold increase in the rate of repair compared to negative control wells, but no difference was observed between resting and exercise supernatants ($P > 0.05$, Figure 5C). Both microvesicle conditions enhanced the closure rate compared to the supernatant conditions, with rMVs stimulating wound healing to a similar extent to that observed in the FBS positive control wells (*i.e.* nearly 3-fold quicker than the negative control), and this process was even more rapid in the presence of exMVs ($P < 0.05$).

Exercise microvesicles stimulate angiogenesis in vitro

Treatment with exMVs induced greater formation of tubule-like structures in comparison to all other conditions ($P < 0.05$, Figure 6A), with no differences observed amongst rMVs and supernatant treatments ($P > 0.05$). The number of branching points was also increased in endothelial cells treated with exMVs compared to those incubated with microvesicle free supernatant ($P < 0.05$, Figure 6B), although exMVs treatment did not increase the number of branching points in comparison to rMVs ($P > 0.5$).

Discussion

This study investigated the time-course of plasma microvesicles appearance in the venous circulation during and after prolonged cycling to gain insights into the relationships between exercise intensity and potential physiological stimuli inducing microvesicle formation *in vivo,* and to establish the physiological relevance of intravascular microvesicles produced during exercise upon human endothelial cells. We have shown, for the first time, that the increase in circulating PMV concentrations occur not only after, but also during exercise within the heavy intensity exercise domain, with a second peak observed at 1 h of recovery suggesting a biphasic response. In addition, exercise within the moderate exercise domain did not elicit a response. The PMV dynamics during exercise moderately correlate with haemodynamic measurements, suggesting that augmented vascular shear stress may be involved in PMV formation in exercising humans. Moreover, we have taken the first step in understanding the effect of microvesicles produced during exercise on the vascular endothelium by demonstrating that exMVs display a stimulatory effect in cultured endothelial cells, revealing a potential link between intravascular microvesicles and vascular adaptation with exercise.

Time-course of microvesicle appearance with moderate and heavy exercise

Earlier attempts to characterize PMV dynamics to exercise generally agree that platelets introduce microvesicles into the circulation during the recovery period, with peak plasma concentrations occurring somewhere within 1 h after the session (11, 36, 48, 49). However, little attention has been placed upon microvesicle temporal kinetics and no study has explored the PMV dynamics during exercise. Our results show for the first time that elevations in PMV concentrations are not limited to the post-exercise recovery period as elevations occurred as early as 30 min into heavy exercise. The concentrations of PMVs increased ~2-fold from baseline during heavy exercise in the present study, which tended to be higher than the 20 and 40 min post-exercise values but similar to values after 1 hour of recovery. The present 60 min recovery data agree with findings of previous studies using traditional flow cytometry in which 2- to 3-fold increases in [PMV] were observed after 1 hour of recovery (11, 48, 49). This increase in PMVs seemed to be related to exercise intensity rather than being a function of the total work done, since PMV concentrations did not increase during moderate exercise even though a greater amount of mechanical work was accomplished by the end of this condition compared to 30 min of heavy cycling (see Supplementary data 2). Moreover, the fact that PMVs increased from baseline and then remained stable from 30 to 60 min during exercise probably indicates a rapid microvesicle turnover, which ought to be confirmed using tracer techniques. Several processes may account for PMV removal as suggested by *in vitro* experiments. Specifically,

evidence for endothelial and renal epithelial cell uptake exists (10, 50). Infused endothelial progenitor cell-derived microvesicles were localised in renal endothelial and tubular cells of rats during recovery from renal injury, suggesting the kidney as a microvesicle extractor organ within these conditions (10). Besides the fact that microvesicles, including PMVs and EMVs, have been identified in the urine of healthy humans (57), it is still difficult to assess the fate of microvesicles with exercise from these experiments, even more so when one considers that renal blood flow (and thus microvesicle delivery) is reduced during intense exercise (51).

The second rise in PMV concentration observed 1 h after the heavy intensity bout is intriguing whilst still in agreement with authors who note peak PMV values between the end of exercise and 1 h (36, 48, 49) or 2 h (11, 48) of recovery. Our biphasic response is a function of the improved temporal resolution of our sampling protocol since previous works did not measure the PMV response during early exercise recovery. Our observations imply a mechanism unrelated to shear rate induces platelet activation after 1 h of exercise and causes an accumulation of PMVs in the circulation comparable to the exercise response. This secondary PMV peak might be related to late platelet recruitment, as exercise may lead not only to platelet activation (12, 15) but also acutely increase platelet concentrations after exercise sessions (15, 27) recruited from various platelet pools including the spleen and bone marrow (18), that then serve as a source of new microvesicles.

EMV concentrations remained similar to baseline across all experimental protocols, implying that the two submaximal exercise stimuli had little impact on endothelial vesiculation. Differing from PMVs, microvesicles derived from endothelial cells have already been studied during exercise, with no changes observed during moderate intensity cycling (40). Inconsistent results exist regarding EMV kinetics during recovery, with researchers reporting increases (31, 34, 48), no change (11, 23, 40, 49), or even decreases (59) after exercise. For instance, Kirk et al. (2013) reported elevated plasma $CD105^+$ and $CD106^+$ EMVs 1.5 h after supramaximal interval cycling. Endoglin (CD105) expression is known to increase in endothelial cells undergoing angiogenesis (41), whereas the expression of vascular adhesion molecule-1 (CD106) is upregulated in the endothelium under inflammatory stimulation (62), which indicates that the strenuous protocol used by Kirk *et al.* (2013) may have caused endothelial activation. The fact that $CD62E^+$ EMVs were elevated in men in a previous study (34) but not in the current experiment is intriguing and may be related to their slightly higher intensity protocol (~70% $\dot{V}O_2$max on an upright cycle ergometer). In agreement, results from our laboratory indicate that EMVs expressing the same markers increase during strenuous exercise conditions (*i.e.* 80% of peak power output under heat stress - EN Wilhelm, J

González-Alonso, ST Chiesa, SJ Trangmar, M Rakobowchuk; unpublished observations), suggesting that higher exercise intensities may be required to induce endothelial vesiculation.

Relationship between platelet microvesicles and putative agonists

The physiological mechanisms eliciting PMV formation during exercise have not been elucidated, and based on previous *in vitro* studies we thought that shear stress might be a potential candidate (39, 44). Hence, a novel finding of this study was the correlation between PMV concentrations and vascular SRs during exercise coupled with a weaker relationship with noradrenaline in these conditions. The latter relationship also seems logical, since sympathetic nervous system activity has been proposed to play a role in haemostasis and catecholamines have been reported to activate platelets (58), with noradrenaline reported to stimulate PMV formation *in vitro* (54). The major role of shear stress on PMV formation *in vivo* still has to be fully elucidated in experiments where shear stress is the single variable manipulated, for example by independently increasing blood flow with the infusion of vasodilators, but the positive associations observed in the current study provides some support for shear stress as a candidate mediator of PMV formation during exercise, with noradrenaline playing a synergistic role within these experimental conditions. The fact that PMVs did not increase markedly during moderate exercise despite substantial increases in shear is interesting and indicates a shear threshold may be required to stimulate PMV formation. Additional mechanisms may involve the purinergic activation of platelets, since increased concentrations of intravascular nucleotides during heavy exercise stimulate platelet activation *ex vivo* (63), but the actual link between circulating PMVs and nucleotides ought to be determined.

Effect of circulating microvesicles on cultured endothelial cells

Evidence from *in vitro* studies outside the context of exercise suggests that microvesicles may be involved in vascular adaptations. Endurance training induces angiogenesis in active tissues in animal (26) and human models (22, 28) which may be stimulated through many mechanisms including hypoxia (16) and local vascular shear stress (21), with VEGF suggested as the major, albeit not the only growth factor implicated (22, 26, 28). Because high intensity training enhances skeletal muscle capillarization (28), and acutely elevates the concentrations of circulating PMVs in humans (as demonstrated in the present study), it seems plausible that microvesicles could play a role in exercise-induced angiogenesis. A unique finding of this study was that exMVs increased the number of endothelial tubule-like formations in comparison to rMVs and their respective microvesicle free supernatants, suggesting that microvesicles produced during exercise have pro-angiogenic potential. The pathways through which exMVs bring about their endothelial effects are unknown, but may

relate to the delivery of VEGF (2, 7) and biologically active lipids (30) to recipient endothelial cells. Furthermore microvesicles obtained from thrombin-stimulated platelets have been shown to display angiogenic potential *in vitro* (7, 30), and *in vivo* (7), which may indicate that PMV could be the microvesicle population stimulating cultured endothelial cells in the present experiments.

Angiogenesis depends on orchestrated endothelial cell migratory and mitogenic events, which in turn are stimulated by growth factors released where new capillaries will infiltrate (22, 28). By staining the proliferation-related protein Ki-67, Jensen et al. (2004) demonstrated that the increased skeletal muscle capillarization observed after training is indeed the result of migration and proliferation of endothelial cells to form new capillaries. Our data indicate that microvesicles may be involved in this intricate process, since exMVs enhanced both endothelial migration and proliferation in individual assays. Endothelial scratch wound-healing (a complex process highly dependent on cell migratory and proliferative capacities) was also elevated with exMV treatment, supporting the findings of individual assays. Previous work outside the context of exercise demonstrates that PMVs induce a dose-dependent proliferation and migration of endothelial cells (30), and may substantiate endothelial wound repair through direct and indirect stimulatory mechanisms (30, 37). It is worth noting that exercise increased the concentration of PMVs in the intravascular space, which might seem counterproductive since chemotactic stimulation on the endothelial apical side would stimulate migration into the vessel lumen. PMVs, however, have been reported to adhere to proteins of the extracellular matrix, serving as a binding site for platelets on the vessel wall at regions of extracellular matrix exposure (38), and platelets have been shown to transmigrate to the subendothelial space under certain conditions (32) where they could deliver MVs to the basal surface of endothelial cells, but the existence of similar mechanisms during exercise are unknown.

Methodological considerations

The current series of studies do have some limitations. It is important to note that correlations do not necessarily represent causation, and the present data only provide indirect evidence of shear stress as a potential stimulus for PMV formation during exercise. Further studies that manipulate shear stress whilst sampling arterial and venous microvesicles across exercising limbs ought to be performed in order to directly evaluate the relevance of shear stress in PMV release with exercise, as well as to address the source of microvesicles (*e.g.* organs or exercising limbs). Current methods for microvesicle isolation do not enable separation of specific populations from human plasma without *ex vivo* stimulation, and even though it seems logical that PMVs were the primary mediators of our findings, we cannot rule out the possibility that other microvesicle populations may be involved.

548 Furthermore, it remains unknown whether the influence of exMVs on endothelial cells resulted
549 simply from their greater concentration, or distinct intrinsic characteristics (*e.g.* different cargo).
550 Finally, it is important to keep in mind that observations taken from static cell culture experiments
551 are not necessarily transferable to other cell lines and to complex whole organism models.

552 *Conclusion*

553 In conclusion, submaximal exercise is a potent physiological stimulus that triggers PMVs formation
554 during and 1 h post exercise, with no impact on EMVs. This phenomenon, however, is exercise
555 intensity dependent and requires a substantial stimulus. The rise in PMVs during exercise was
556 coupled with changes in vascular shear stress, and plasma noradrenaline concentration, with both
557 variables explaining part of microvesicle dynamics during exercise. Moreover, human circulating
558 microvesicles produced during exercise increased the angiogenic potential in cultured endothelial
559 cells, which is supported by their mitogenic and chemotactic enhancements. Together, this set of
560 integrative physiology experiments in healthy young humans characterised the physiological time-
561 course of PMV and EMV appearance during and after submaximal exercise, and provides the first
562 evidence that exercise-derived microvesicles play an important biological role within the human
563 vasculature, suggesting a novel mechanism that may help us to further understand how exercise
564 mediates vascular adaptation.

Competing interests

All authors declare no conflict of interests in relation to this work.

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Author contributions

Experiments were performed in the Centre for Sport, Exercise and Rehabilitation, Brunel University London, UK. E.N.W., M.R. and J.G.-A. were involved in the design and conception of the studies. E.W.N. and M.R. were involved in data collection and analysis of *in vivo* and *in vitro* experiments. E.W.N, M.R. and C.P. optimized the methods to quantify the microvesicles. E.W.N, M.R. and J.G-A were involved with interpretation of results, writing, as well as reviewing the manuscript. The final version of the manuscript was approved by all authors before submission.

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Table 1. Limb haemodynamics at baseline and throughout resting control or exercise and recovery from moderate or heavy semi-recumbent cycling.

	Experimental Condition									
	Baseline	Rest or exercise		Recovery						
Time (min)	**0**	**20**	**40**	**65**	**80**	**100**	**120**	**150**	**180**	**240**
BA diameter (cm)										
Control	0.39±0.01	0.39±0.02	0.39±0.02	0.39±0.01	0.39±0.01	0.39±0.02	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01
Moderate exercise	0.39±0.01	0.41±0.01*†	0.41±0.01*†	0.40±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01
Heavy exercise	0.38±0.02	0.41±0.01*†	0.42±0.01*†	0.41±0.01*†	0.40±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.04
BA Vmean (cm / s)										
Control	9.4±0.9	8.8±0.9	8.8±1.1	9.3±1.0	8.3±1.0	8.7±0.6	8.4±0.8	8.4±0.7	8.3±0.9	8.3±1.1
Moderate exercise	10.1±1.2	19.4±2.6*†#	20.5±3.4*†#	16.0±2.6	10.9±2.0	9.3±1.3	8.7±1.5	8.2±1.6	7.9±1.2	7.9±1.1
Heavy exercise	10.4±1.3	30.5±2.8*†	32.3±3.3*†	23.7±3.6*†	12.9±2.5	11.6±2.0	11.5±1.9	9.3±1.6	9.9±1.4	9.0±1.3
BA mean shear rate (/ s)										
Control	98±10	92±10	93±13	97±13	88±13	91±8	88±10	88±10	87±11	87±13
Moderate exercise	106±15	194±28*†#	201±36*†#	162±29	112±21	97±16	91±18	88±19	84±15	83±14
Heavy exercise	112±18	300±32*†	309±34*†	236±40*†	135±29	122±24	120±22	97±19	106±19	97±17
FA diameter (cm)										
Control	0.90±0.02	-	-	0.89±0.02	0.90±0.02	0.89±0.02	0.89±0.02	0.89±0.02	0.89±0.02	0.90±0.02
Moderate exercise	0.90±0.02	-	-	0.96±0.02*†	0.94±0.02†	0.92±0.02	0.92±0.02	0.91±0.02	0.91±0.02	0.91±0.02
Heavy exercise	0.90±0.02	-	-	0.96±0.02*†	0.96±0.02†	0.92±0.02	0.91±0.02	0.91±0.02	0.90±0.02	0.90±0.02
FA Vmean (cm / s)										
Control	10.0±0.9	-	-	9.1±1.1	9.3±0.8	8.5±1.0	9.3±0.9	9.3±1.0	8.9±0.7	8.8±0.7
Moderate exercise	8.2±0.5	-	-	16.6±1.4*†#	9.0±0.7	8.8±0.5	8.2±0.4	9.0±0.7	8.5±0.7	9.0±0.4
Heavy exercise	9.0±0.7	-	-	29.4±2.0*†	12.1±1.0†	10.5±0.9	10.4±0.9	9.5±0.7	9.6±0.9	9.4±0.6
FA mean shear rate (/ s)										
Control	45±5	-	-	42±6	42±4	39±5	42±5	42±5	41±4	40±4
Moderate exercise	37±2	-	-	70±7*†#	39±3	39±3#	36±2	40±4	38±4	40±2
Heavy exercise	40±4	-	-	122±8*†	52±5	46±5	46±4	42±4	43±4	42±3

Data are mean±SEM for 9 participants. BA, brachial artery; Vmean, time averaged mean blood flow velocity; FA, femoral artery; * $P \leq 0.05$ compared to baseline within condition; † $P \leq 0.05$ from control visit at the same time-point; # $P \leq 0.05$ from heavy exercise visit at the same time-point.

Table 2. Central haemodynamics at baseline and throughout resting control or exercise and recovery from moderate or heavy semi-recumbent cycling.

	Experimental Condition									
	Baseline	Rest or exercise		Recovery						
Time (min)	**0**	**20**	**40**	**65**	**80**	**100**	**120**	**150**	**180**	**240**
Heart rate (beats / min)										
Control	59±4	61±4	60±3	61±2	58±3	58±3	59±3	57±3	59±3	60±4
Moderate exercise	59±3	118±5*†#	118±6*†#	78±7*	63±7	65±3	61±3	58±3	56±2	57±2
Heavy exercise	61±3	157±5*†	158±4*†	84±3*†	73±2†	64±3	62±3	60±3	60±4	58±2
Left ventricular EDV (ml)										
Control	132±9	130±8	130±8	129±9	127±8	129±7	131±8	131±8	124±8	128±9
Moderate exercise	136±9	145±11	142±9	134±9	131±10	131±8	130±9	134±9	130±8	131±9
Heavy exercise	126±10	146±12	143±12	122±8	120±8	120±9	126±10	128±10	124±9	127±10
Left ventricular ESV (ml)										
Control	45±6	46±6	44±5	45±5	45±5	44±5	45±5	46±5	41±5	44±6
Moderate exercise	53±8	43±7	41±6*	47±6	47±6	49±6	49±7	50±6#	47±6	48±7
Heavy exercise	46±7	27±7	27±7	35±2	38±3	39±5	44±7	44±6	45±9	43±6
Stroke volume (ml)										
Control	92±4	86±3	89±4	89±4	87±4	89±3	89±4	88±3	87±3	89±4
Moderate exercise	86±3	107±6*†	107±5*†	96±5	86±3	84±2	83±3†	84±3	84±3	88±4
Heavy exercise	89±5	119±6*†	116±5*†	79±10	82±3	80±2†	82±2†	90±5	83±3†	89±5
SBP (mmHg)										
Control	130±2	127±2	127±3	127±4	129±4	129±4	128±4	124±4	123±4	126±4
Moderate exercise	130±3	160±3*†#	153±4*†#	134±3	125±2	125±4	127±4	129±3#	125±4	129±3
Heavy exercise	129±2	173±3*†	176±3*†	123±5	119±2	121±3	121±2	120±3	119±3	122±3
DBP (mmHg)										
Control	70±2	69±3	69±3	70±4	71±3	71±3	73±3	71±3	71±3	71±3
Moderate exercise	71±1	79±2*	77±2*	74±3#	73±2	72±3	72±3	73±2	70±2	74±2
Heavy exercise	68±2	80±2*	80±3*	65±2	71±1	72±2	72±2	70±2	70±2	69±2

Data are mean±SEM for 5-9 participants; EDV: end diastolic volume (n = 5); ESV: end systolic volume (n = 5); SBP: systolic blood pressure; DBP: diastolic blood pressure; *: $P \leq 0.05$ compared to baseline within condition; †: $P \leq 0.05$ from control trial at the same time-point; #: $P \leq 0.05$ from heavy exercise trial at the same time-point.

Table 3. Blood-derived parameters at baseline and throughout resting control or exercise and recovery from moderate or heavy semi-recumbent cycling.

	Experimental Condition								
	Baseline	Rest or exercise		Recovery					
Time (min)	**0**	**30**	**60**	**80**	**100**	**120**	**150**	**180**	**240**
Haematocrit (%)									
Control	42.1±0.8	42.2±0.9	42.2±0.9	42.1±0.9	42.3±0.8	42.7±0.8	42.9±0.9	42.3±0.9	42.6±0.9
Moderate exercise	42.2±0.8	44.3±0.6	44.6±0.7*	43.3±0.7*	42.9±0.7	42.9±0.4	43.2±0.5	43.1±0.7	43.0±0.8
Heavy exercise	42.3±0.9	45.7±0.9*†	45.2±0.8*†	43.7±1.1†	42.9±0.9	42.6±0.9	42.6±0.9	42.8±0.9	42.9±0.8
Haemoglobin (g / l)									
Control	141±4	142±3	141±4	141±3	140±3	142±3	142±4	140±4	142±4
Moderate exercise	144±3	154±3*†	154±3*	146±3	146±3	146±3	146±3	148±3	145±3
Heavy exercise	142±3	155±4*†	154±4*†	147±4	141±3	139±4	142±3	142±3	142±3
Plasma volume change (%)									
Control	-	-0.4±0.7	-0.1±1.4	0.3±1.4	0.5±.1.7	-1.3±1.9	-1.7±1.6	0.7±1.6	-0.9±1.4
Moderate exercise	-	-10.2±1.2*	-10.7±1.4*	-3.5±1.2	-2.8±1.2	-2.4±2.5	-2.8±2.4	-4.3±2.2	-2.2±2.1
Heavy exercise	-	-13.7±1.2*	-13.3±1.5*	-5.3±1.7*	-0.3±1.9	2.0±2.5	-0.3±1.7	-0.6±1.5	-1.1±1.3
Lactate (mmol / l)									
Control	1.0±0.1	0.9±0.1	0.9±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.8±0.1
Moderate exercise	1.0±0.1	1.6±0.1*#†	1.3±0.1#†	0.9±0.1#	0.8±0.1#	0.8±0.1#	0.7±0.1#	0.8±0.1	1.1±0.1
Heavy exercise	1.0±0.1	6.3±0.7*†	5.1±0.9*†	2.2±0.3*†	1.4±0.2	1.2±0.1	0.9±0.1	0.9±0.1	1.0±0.1
Noradrenaline (nmol / l)									
Control	3.2±0.3	3.2±0.5	3.6±0.4	-	-	2.9±0.4	-	2.6±0.3	3.4±0.4
Moderate exercise	3.0±0.3	4.4±0.8#	4.4±0.5	-	-	2.5±0.4	-	3.7±0.6	3.0±0.5
Heavy exercise	3.0±0.2	8.8±1.1*†	9.0±1.5*†	-	-	2.8±0.4	-	3.0±0.5	3.5±0.6
IL-6 (pg / ml)									
Control	0.8±0.1	0.8±0.2	0.7±0.2	-	-	1.1±0.3	-	2.0±0.5*	2.5±0.5*
Moderate exercise	0.8±0.2	0.7±0.1	0.9±0.1#	-	-	1.3±0.2	-	1.7±0.2*	1.9±0.5*
Heavy exercise	0.6±0.1	0.7±0.1	1.9±0.3*†	-	-	2.0±0.4*	-	2.3±0.3*	1.8±0.1*

Data are mean±SEM for 9 participants; IL-6: interleukin-6 *: $P \leq 0.05$ compared to baseline within condition; †: $P \leq 0.05$ from control trial at the same time-point; #: $P \leq 0.05$ from heavy exercise trial at the same time-point.

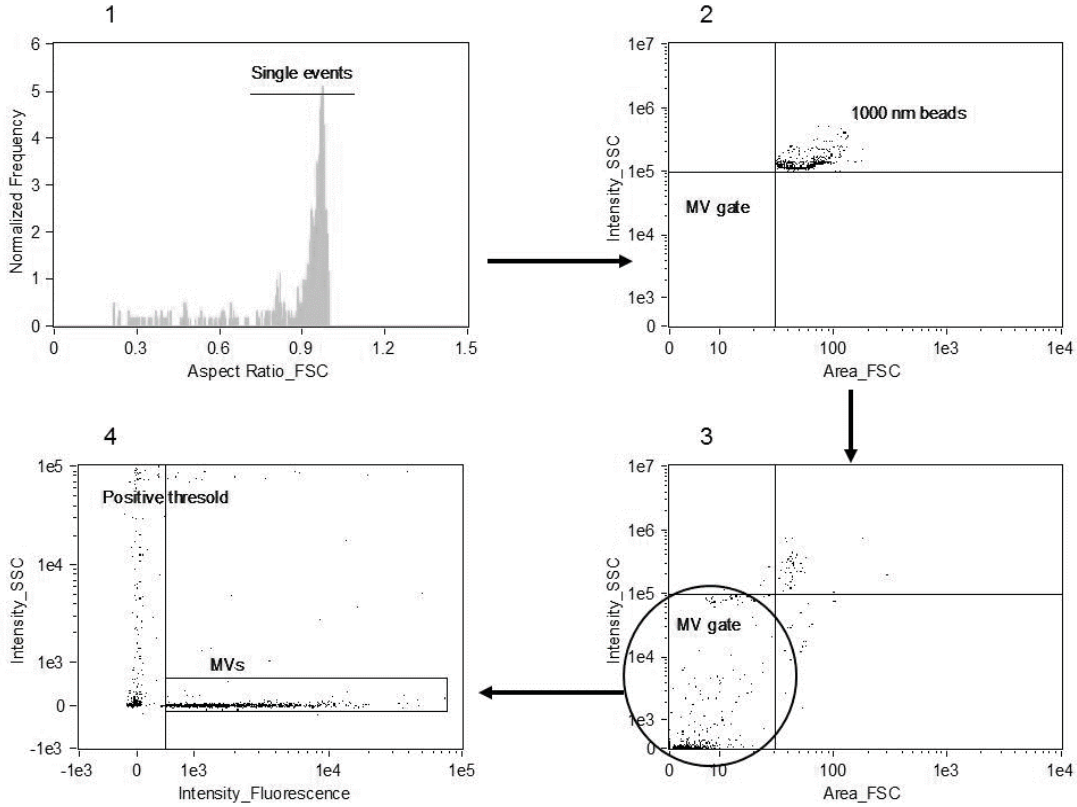

Figure 1. Microvesicle gating strategy. Single events are separated from doublets through their high aspect ratio values (1), and forward scatter (FSC) by side scatter (SSC) do-plots of calibration beads are used to determine a 1 µm size gate (2) which is applied in platelet poor plasma samples to exclude non-microvesicle large events (3). Using the ImageStream microscopy feature at 60x magnification, microvesicles tend to exhibit low SSC intensity and moderate fluorescence for the relevant conjugated antibody (4).

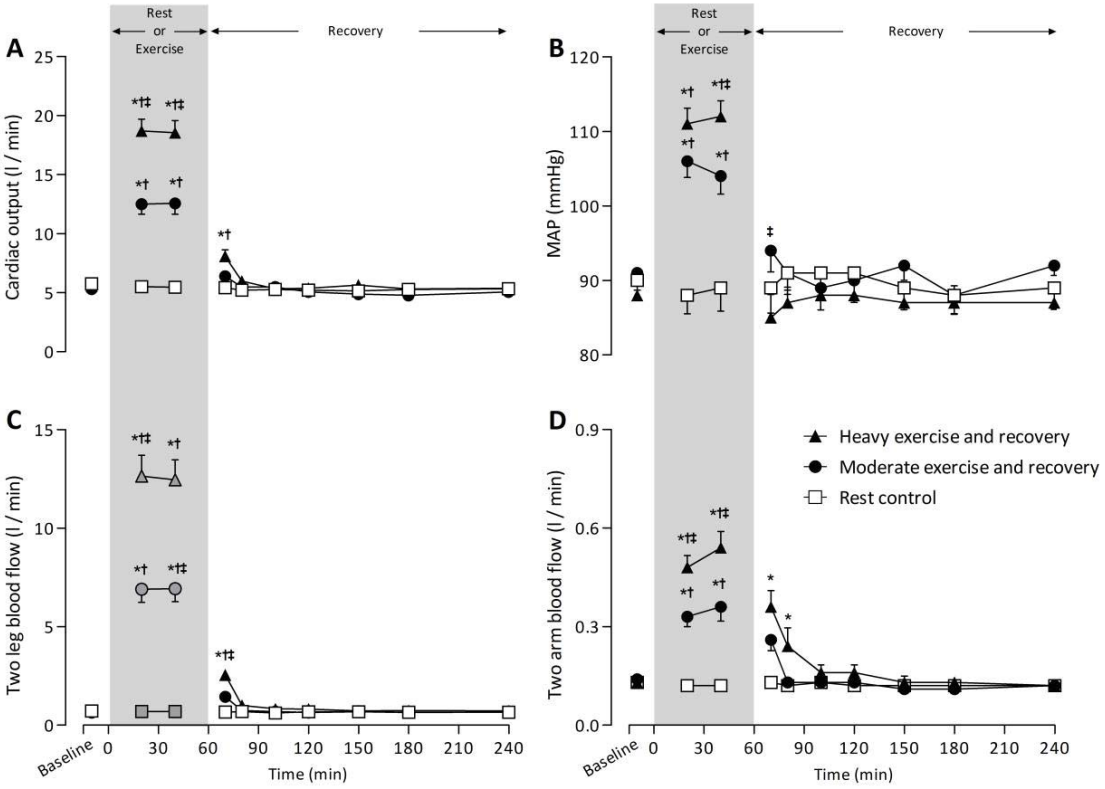

Figure 2. Systemic and limb haemodynamics during the experimental trials with significant increases during exercise and into early recovery. Leg blood flow reflecting exercise time-points (grey symbols) were estimations. Data are expressed as means ± SEM; n = 9; * Significant difference from baseline within condition ($P < 0.05$); † Significant difference from control visit ($P < 0.05$); ‡ Significant difference from moderate exercise ($P < 0.05$).

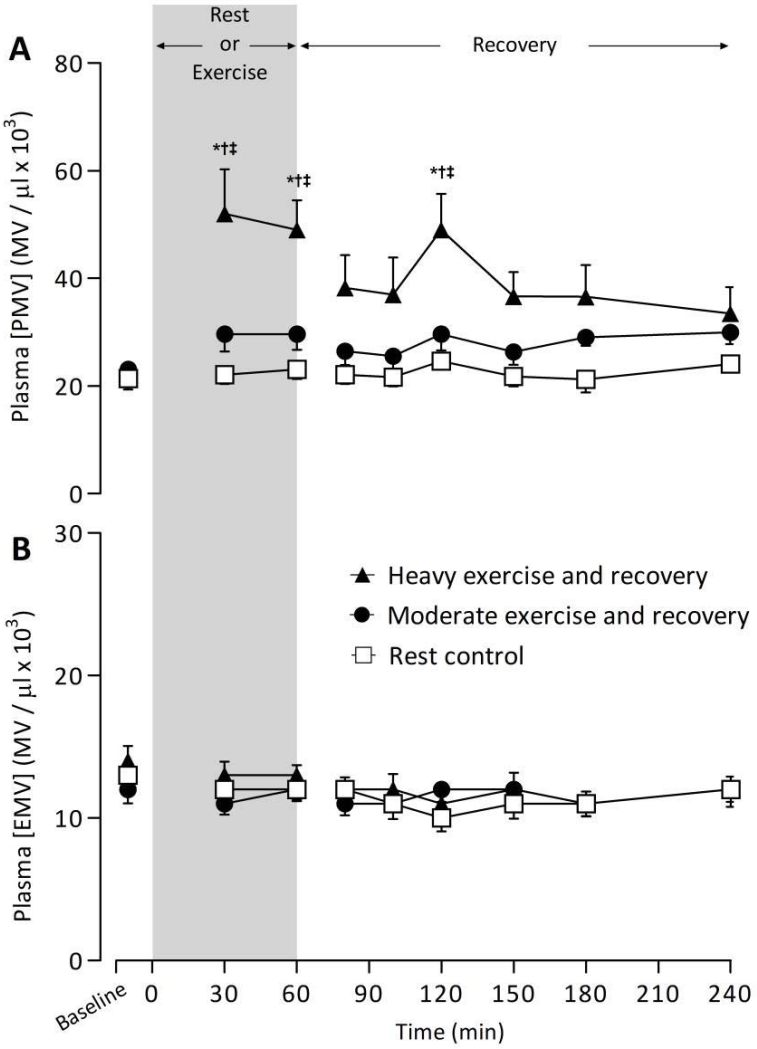

Figure 3. Circulating platelet (PMV) (A) and endothelial (EMV) (B) microvesicle concentrations during the experimental trials. Exercise had no impact on EMV concentrations, but heavy intensity cycling increased circulating PMVs during and at 1 h of post-exercise recovery. Data are expressed as means ± SEM; n = 9; * Significant difference from baseline ($P < 0.05$); † Significant difference from control visit ($P < 0.05$); ‡ Significant difference from moderate exercise ($P < 0.05$).

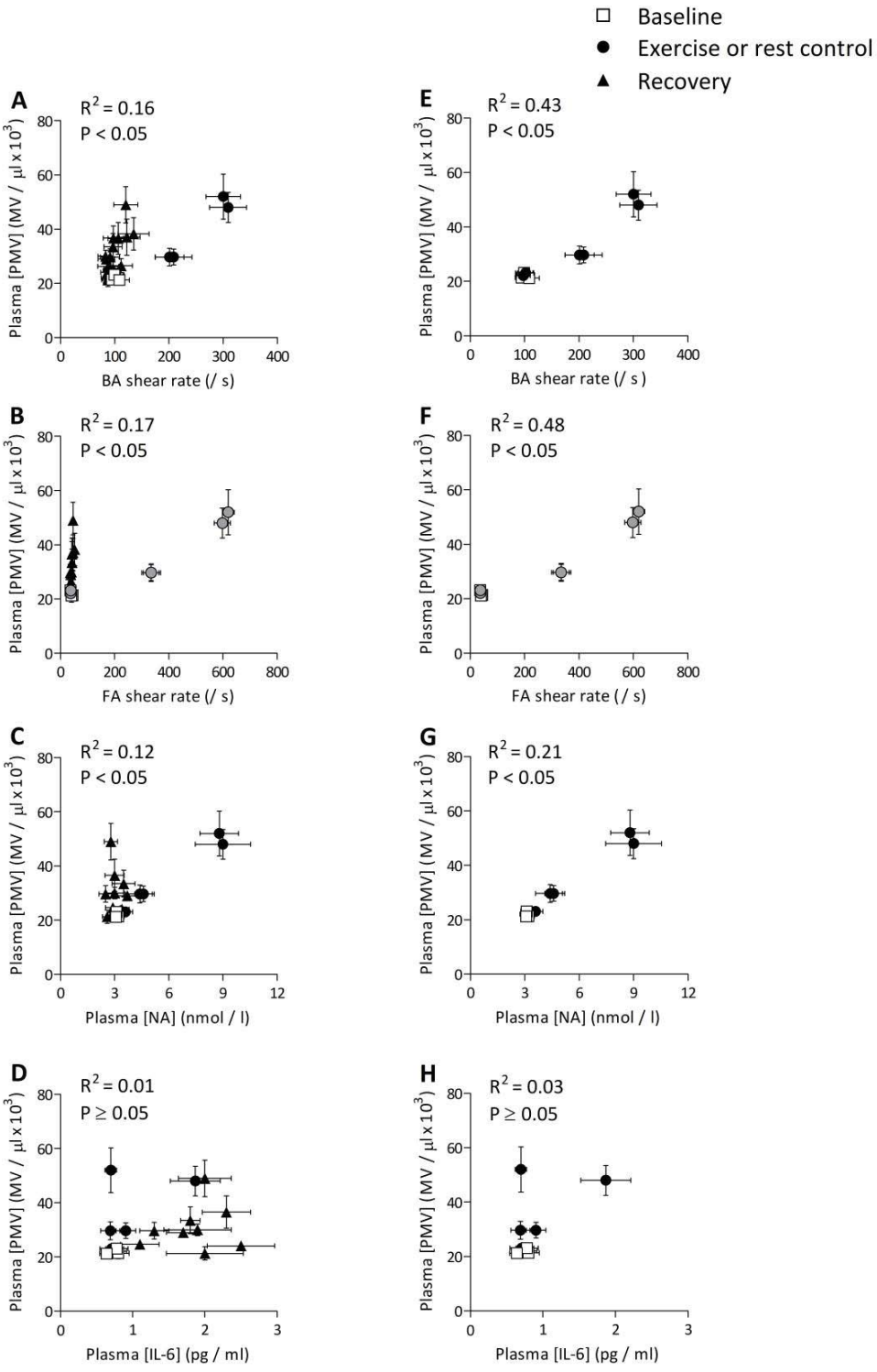

Figure 4. Relationships between platelet microvesicle (PMV), vascular shear rate, and biochemical variables measured at all time-points (A-D), and only during baseline and exercise (E-H). Exercising limb shear rate was estimated during exercise (grey symbols), yet a positive correlation was observed between exercise PMVs and FA shear rate (F). Data are expressed as means ± SEM. BA, brachial artery; FA, femoral artery; NA, noradrenaline; IL-6, interleukin-6.

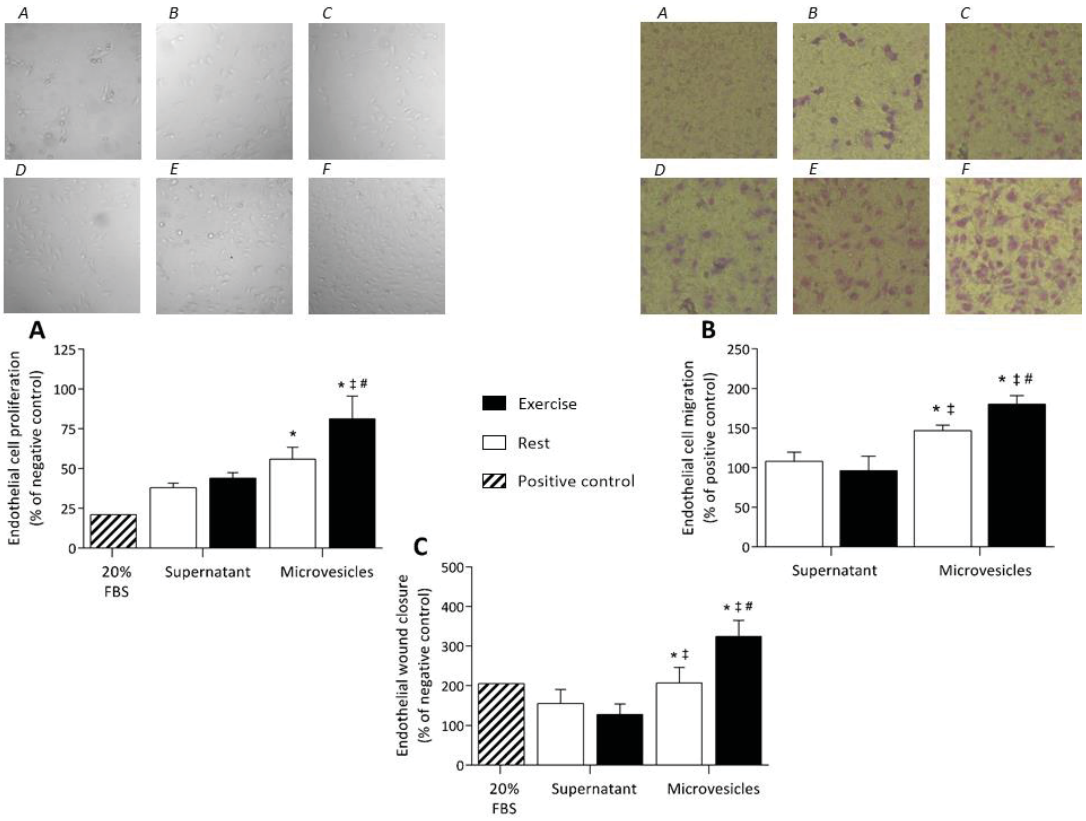

Figure 5. Human umbilical vein endothelial cell proliferation (A), migration (B), and scratch wound-healing (C) in response to resting and exercise microvesicle or microvesicle free supernatant treatment with significantly greater proliferative, migratory and wound closure rates when microvesicles were within the medium and a potentiated effect when microvesicles were derived from exercise plasma samples. Above the graphs are representative proliferation (top left) and migration (top right) images of negative control (A), positive control (B), rest microvesicle free supernatant (C), exercise microvesicle free supernatant (D), rest microvesicle (E), and exercise microvesicle (F) treatments. Data are expressed as means ± SEM; n = 5 for all assays; * Significant difference from rest supernatant ($P < 0.05$); ‡ Significant difference from exercise supernatant ($P < 0.05$); # Significant difference from rest microvesicles ($P < 0.05$).

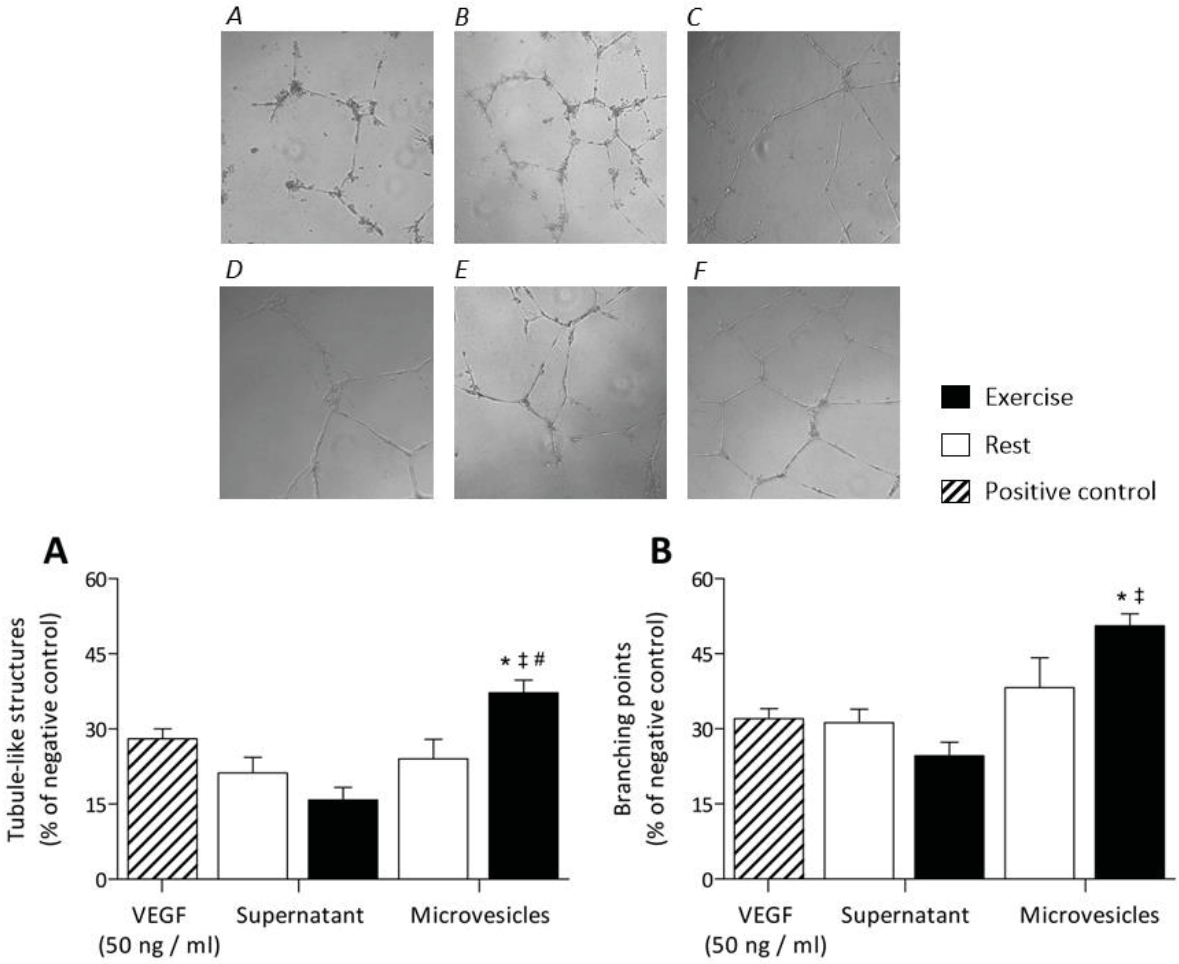

Figure 6. *In vitro* angiogenesis determined by formation of tubule-like structures (A) and branching points (B) of human umbilical vein endothelial cells in response to resting and exercise microvesicle or microvesicle free supernatant treatment. Exercise-derived microvesicles increased the formation of tubule-like structures (A) in comparison to rest microvesicles and microvesicle free supernatants; and also increased the number of branching points (B) in comparison supernatant treatments. Above the graphs are representative images of negative control (A), positive control (B), rest microvesicle free supernatant (C), exercise microvesicle free supernatant (D), rest microvesicle (E), and exercise microvesicle (F) treatments. Data are expressed as means ± SEM; n = 5; * Significant difference from rest supernatant ($P < 0.05$); ‡ Significant difference from exercise supernatant ($P < 0.05$); # Significant difference from rest microvesicles ($P < 0.05$).